Overexpression of a Hyperpolarization-Activated Cation Current (I_h) Channel Gene Modifies the Firing Activity of Identified Motor Neurons in a Small Neural Network

Ying Zhang, Ricardo Oliva, Günter Gisselmann, Hanns Hatt, John Guckenheimer, and Ronald M. Harris-Warrick

The hyperpolarization-activated cation current (I_h) is widely distributed in excitable cells. I_h plays important roles in regulation of cellular excitability, rhythmic activity, and synaptic function. We previously showed that, in pyloric dilator (PD) neurons of the stomatogastric ganglion (STG) of spiny lobsters, I_h can be endogenously upregulated to compensate for artificial overexpression of the Shal transient potassium channel; this maintains normal firing properties of the neuron despite large increases in potassium current. To further explore the function of I_h in the pyloric network, we injected cRNA of PAIH, a lobster gene that encodes I_h, into rhythmically active PD neurons. Overexpression of PAIH produced a fourfold increase in I_h, although with somewhat different biophysical properties than the endogenous current. Compared with the endogenous I_h, the voltage for half-maximal activation of the PAIH-evoked current was depolarized by 10 mV, and its activation kinetics were significantly faster. This increase in I_h did not affect the expression of I_o or other outward currents. Instead, it significantly altered the firing properties of the PD neurons. Increased I_h depolarized the minimum membrane potential of the cell, reduced the oscillation amplitude, decreased the time to the first spike, and increased the duty cycle and number of action potentials per burst. We used both dynamic-clamp experiments, injecting the modeled PAIH currents into PD cells in a functioning STG, and a theoretical model of a two-cell network to demonstrate that the increased I_h was sufficient to cause the observed changes in the PD activity.

Key words: I_h; I_o; STG; computational modeling; gene expression; neuronal network

Introduction

The hyperpolarization-activated cation current (I_h) was first identified in cardiac sinoatrial node cells as a pacemaker current (Noma and Irisawa, 1976; Brown and Difrancesco, 1980; Yanagihara and Irisawa, 1980). I_h has now been found in many cell types in the CNS (Difrancesco, 1993; Pape, 1996). Besides playing important roles in controlling rhythmic activity in the heart (Noma and Irisawa, 1976; Brown and Difrancesco, 1980; Yanagihara and Irisawa, 1980) and the brain (Pape and McCormick, 1989; Bal and McCormick, 1997; Luthi and McCormick, 1998), I_h also regulates cellular excitability (Pape and McCormick, 1989; Thoby-Brisson et al., 2000), synaptic transmission and plasticity (Beaumont and Zucker, 2000; Mellor et al., 2002), and dendritic integration (Poo los et al., 2002) in different preparations. Four I_h channel genes, HCN1 to HCN4, have been cloned in mammals (Santoro et al., 1997, 1998; Ludwig et al., 1998). When expressed in Xenopus oocytes or heterologous cell lines, HCN1- to HCN4-expressing currents show very different biophysical properties. HCN channels can also interact with auxiliary proteins such as minK-related protein (MinK-R or KCNE2) (Yu et al., 2001), which can further contribute to the diversity of H-currents in vivo.

Despite this molecular knowledge, our understanding of the functional roles of I_h within the context of intact neural networks is still uncertain (Vassalle, 1995; Chevaleyre and Castillo, 2002; Miale et al., 2002). The complexity of vertebrate systems makes these system-level studies difficult. In our study, we took advantage of a small and clearly defined network, the 14-neuron pyloric network in the stomatogastric ganglion (STG) of the spiny lobster, Panulirus interruptus. In this system, we can manipulate the expression of single genes in a single identified neuron. This allows us to study the function of the channel both in individual neurons and in the intact, functioning network. I_h is present in STG neurons (Golowash and Marder, 1992; Kiehn and Harris-Warrick, 1992), and can be modulated by dopamine (Harris-Warrick et al., 1995), red pigment-concentrating hormone (Dickinson et al., 2001), and serotonin (Kiehn and Harris-Warrick, 1992).

One possible physiological role of I_h is to regulate postinhibitory rebound that drives resumption of bursting after synaptic inhibition in the pyloric rhythm. We previously showed that postinhibitory rebound after hyperpolarizing current injections is shaped by a complex opposition between I_h and the transient...
potassium current, $I_{K}$ (Kiehn and Harris-Warrick, 1992). Recent work from our lab suggests that $I_{K}$ is involved at the molecular level in balancing the activity of the $I_{A}$ gene, shal, in pyloric neurons (MacLean et al., 2003). Overexpression of shal by RNA injection into pyloric dilator (PD) neurons produced a very large $I_{A}$, but did not significantly change the firing properties of the neurons. This is attributable to an activity-independent compensatory increase in $I_{K}$ that accompanied the shal-induced overexpression of $I_{A}$, thus maintaining the normal activity of the network. We asked whether this compensatory response is bidirectional: does overexpression of the $I_{A}$ protein upregulate $I_{K}$ as well?

PAIH, a gene encoding an $I_{A}$ channel, has been cloned from the closely related spiny lobster Panulirus argus (Gisselmann et al., 2003). In our present study, we overexpressed PAIH in PD neurons by direct microinjection of its RNA. Overexpression of PAIH produced very large increases in $I_{A}$ in PD neurons. However, no compensatory enhancement of $I_{K}$ or other outward currents was detected. Instead, it significantly changed the firing properties of the injected PD neurons. These experimental results were confirmed by dynamic-clamp experiments, injecting an artificial PAIH current into PD cells, and a theoretical model of a PD neuron in a simplified oscillating network.

Materials and Methods

STG dissection and PD cell identification

Pacific spiny lobsters (P. interruptus) were purchased from Don Tomlinson Commercial Fishing (San Diego, CA) and maintained in artificial seawater at 16°C until use. Animals were anesthetized by cooling on ice for 30 min before dissection. The STG was dissected along with its motor nerves and associated commissural and esophageal ganglia (Mulloney and Silverston, 1974), and pinned in a Sylgard-lined dish. The preparation was superfused continuously (3 ml/min) with saline (16°C) containing (in mM): 479 NaCl, 12.8 KCl, 13.7 CaCl$_2$, 3.9 Na$_2$SO$_4$, 10.0 MgSO$_4$, 2 glucose, and 11.1 Tris base, pH 7.35 (Mulloney and Silverston, 1974). Extracellular recordings were made from identified motor nerves using glass suction electrodes. Individual somata were impaled with glass microelectrodes (10–25 MΩ). Individual spikes were identified by their typical shape and recorded extracellularly on the PD motor nerve and by their typical shape and recorded intracellularly on the PD neuron in a simplified oscillating network.

RNA microinjection into neurons

The PAIH clone in a pTracer2 vector was obtained from H.H.; the accession number is GenBank AY280847. PAIH DNA was linearized with XhoI and transcribed in vitro using a T7 mMessage mMachine kit (Ambion, Austin, TX). The transcripts were cleaned using the RNeasy mini kit (Qiagen, Valencia, CA). After neuronal identification, PD neurons were injected with an RNA solution using pressure pulses (40 psi; 0.2 Hz) driven by a homemade pressure injector and a pulse generator (Master-8; AMPI, Jerusalem, Israel). The RNA solution contained 0.4–0.7 pg/μl PAIH and 0.04% Fast Green to monitor the injection. PD cells were injected with approximately equivalent amounts of RNA based on the color of the coinjected Fast Green. Fast Green alone was injected into control neurons, which were otherwise treated identically to the RNA-injected neurons. After injection, the ganglion, with intact connections to the commissural and esophageal ganglia, was incubated in sterilized recording saline without Tris base but containing 5 mM HEPES, pH 7.4, 2 gm/l glucose, 50,000 U/l penicillin, and 50 mg/l streptomycin at 16°C for 5 d to allow the expression of the proteins.

Electrophysiological analysis of currents

PD neurons. After 5 d in organ culture, PD neurons were recorded intracellularly and voltage clamped using an Axoclamp 2B amplifier driven by pClamp8 software (Axon Instruments, Foster City, CA). Microelectrodes were filled with 3 M KCl and had a tip resistance of 14–20 MΩ for voltage recording and ≤8 MΩ for current injection. To isolate PD neurons from most synaptic input, we superfused the ganglion with saline containing 10−7 M tetrodotoxin and 5 × 10−6 M picROTOX. The currents of interest were additionally isolated from most other currents with the following channel blockers: for $I_{K}$, 2 × 10−4 M CdCl$_2$ (to block calcium and calcium-activated currents), 2 × 10−2 M tetrodahammolino chloride (TEA) (to block most potassium currents), and 4 × 10−3 M 4-aminopyridine (to block $I_{K}$); for $I_{A}$, 2 × 10−4 M CdCl$_2$, 5 × 10−3 M CaCl$_2$ (to block $I_{K}$), and 2 × 10−2 M TEA; for nonactivating outward currents [Ca$^{2+}$-activated potassium current ($I_{K(Ca)}$) and delayed rectifiers ($I_{K(VO)}$), 5 × 10−3 M CaCl$_2$; the neuron was held at −40 mV, where $I_{A}$ is almost completely inactivated in PD cells.

To measure $I_{K}$, the cells were held at −40 mV, and the voltage dependence of activation was measured with a series of 8 sec hyperpolarizing voltage steps at 20 sec intervals from −50 to −130 mV in 10 mV increments. These steps were not leak subtracted. The reversal potential of $I_{K}$ was measured from the tail currents after a deactivating pulse to −100 mV for 8 sec with a series of 4 sec pulses from −60 to −20 mV in 5 mV increments.

To measure the peak currents of $I_{K}$, the cells were held at −50 mV and depolarized to +20 mV for 400 msec after a deactivating prepulse to −120 mV for 400 msec. Traces were leak subtracted using a P/6 protocol with steps opposite to the sign of activation.

To measure the non-$I_{A}$ potassium currents (a mixture of $I_{K(Ca)}$ and $I_{K(VO)}$), the cell was held at −40 mV. A series of 400 msec voltage steps were delivered from −40 mV to +30 mV with 10 mV increments. The traces were leak subtracted using a P/6 protocol.

Current analysis. $I_{K}$ amplitudes were measured from single exponential fits of the data performed in Clampfit, version 8.1 (Axon Instruments), extrapolated back to the beginning of the hyperpolarizing step and forward to approximate the steady state at 12 sec. Currents were converted to conductances using a reversal potential for $I_{K}$ ($V_{rev}$) of −30 mV, which we determined from analysis of the tail currents. The conductance–voltage data were fit to the Boltzmann equation:

$$g = g_{max} \frac{1}{1 + e^{\left(V_{rev} - V / s\right)}}$$

where $g$ is the conductance, $g_{max}$ is the maximal conductance, $V_{rev}$ is the voltage of half-activation, and $s$ is the slope factor.

Analysis of rhythmic activity

We analyzed rhythmic activity in PD neurons using Spike2 (Cambridge Electronic Design, Cambridge, UK). The period was the time between the first spike of two adjacent oscillations, and the frequency was the inverse of the period. The minimal membrane potential ($V_{min}$) was measured at the most hyperpolarized potential in the trough of the oscillation. The oscillation amplitude was the difference between $V_{min}$ and the most depolarized potential of the slow wave oscillation (at the base of the action potentials). The time to the first spike was the time from $V_{min}$ to the top of the first spike.

Dynamic-clamp studies

We used the dynamic clamp (Sharp et al., 1993a,b) to inject an artificial $I_{A}$ into PD neurons during their normal oscillating firing pattern. The membrane potential $V_{m}$ recorded with a microelectrode in the PD cell body was fed into a Digidata 1200A board (Axon Instruments) and digitized at 2 kHz with in-house software that was kindly modified by Dr. A. A. Prinz (Brandeis University, Waltham, MA) from a C++ program written by Dr. R. D. Pinto (Physics Institute, University of São Paulo, São Paulo, Brazil) (Pinto et al., 2001). The dynamic-clamp software calculated the $I_{A}$ that would be active given a set of model parameters and $V_{m}$, and the injected current was calculated as follows:

$$I_{inj} = g_{max}m(V - V_{rev})$$

where $m$ changed according to $dm/dt = (m_a - m)/\tau_m$ and $m_a$ was given by $m_a = 1/(1 + \exp((V_m - V_{rev}))/\tau_{delay}))$. The parameters $g_{max}$, $V_{rev}$, $V_{min}$, and $\tau_{delay}$ were derived from electrophysiological recordings of $I_{A}$ in PAIH-injected PD neurons. The activities of the neurons during dynamic clamp were recorded on a separate computer using Axoscope (Axon Instruments).
Student’s t tests were used to assess statistical significance. Throughout this paper, all of the calculated values are reported as means ± SD.

Modeling

We investigated how an increase in \( I_h \) affects the firing properties of PD neurons using a mathematical model of these cells. The model is based on a previous model of the lateral pyloric (LP) neuron of the crab (Buchholz et al., 1992; Golowash and Marder, 1992). The model incorporates the known relevant currents for the PD neurons in the STG: Hodgkin–Huxley-type currents for sodium (\( I_{Na} \)), calcium (\( I_{Ca} \)), a delayed-rectifier potassium current (\( I_{K(V)} \)), a calcium-activated potassium current (\( I_{K(Ca)} \)), a transient potassium current (\( I_{K} \)), and a leak current (\( I_{leak} \)). Parameters for \( I_{Ca}, I_{K(V)}, I_{K(Ca)}, I_{K}, I_{leak} \) were obtained from our previous work (Kloppenburg et al., 1999; Willms et al., 1999; Johnson et al., 2003). The experimentally induced H-current (\( I_h \)) was modeled as a constant conductance parameter \( g_h \). We use this simple representation of \( I_h \), because the activation time constant at physiological voltages is much slower than the time scales of oscillations examined in our model simulations. During any simulation with a model that included a voltage-dependent H-current with its measured time constants, changes in its conductance would be negligible.

Our modeled PD neuron is electrically coupled to a second cell that embodies the rest of the pyloric network that normally drives the PD cell into bursting. The parameters in our model PD cell have been tuned to reflect values experimentally measured, whereas the parameter values of the driver cell were chosen to produce a bursting mode characteristic of the network oscillations that normally drive the PD neuron. The coupling coefficients were chosen so that, in the absence of \( I_h \), the PD cell follows the bursting pattern of the driver cell. In addition, the coupling was set to be asymmetric. This asymmetrically coupled two-cell model is a surrogate for a more detailed model that would represent each of the individual neurons in the pyloric circuit and include chemical as well as electrical synapses. Although in the pyloric network the strongest electrical coupling of each PD cell is its electrical coupling to the other PD cell, we hypothesize that modification of the driven PD cell will have a smaller feedback to the remainder of the network than the input it receives from the network. In the absence of more detailed models, this hypothesis has not been tested.

The equations of the model are shown in Table 1. Quantities with subscript 1 correspond to the driver cell, and quantities with subscript 2 correspond to the modified PD cell. The constant parameters in the model are listed in Table 2. Under control conditions, \( I_h \) in the PD cell is very small, and we include it in our calculations of the leak current. Therefore, we set \( g_h \) equal to 0. Our model assumes that the effects of any \( I_h \) in the remainder of the network are reflected in the leak current of the PD cell. We then model the experimentally induced \( I_h \) in the PD cell by setting \( g_h \) to a value similar to the increase in \( I_h \) seen in the \( PAIH \) injection experiments.

The differential equations for the model were numerically integrated using the Radau integrator (Hairer and Wanner, 1991) for a time span of 18 sec. This time span was sufficient for transients to die out, and the time interval between the minimum voltage points on the \( V(t) \) curve during the last two cycles of the PD cell oscillations was selected for analysis. The oscillation frequency, duty cycle, oscillation amplitude, minimum volt-

### Table 2. Parameter for the modeled cells

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
<th>Value</th>
<th>Units</th>
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<tr>
<td>( C_f )</td>
<td>Membrane capacitance</td>
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<td>nF</td>
</tr>
<tr>
<td>( I_{ext} )</td>
<td>External current</td>
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<td>( \mu A )</td>
</tr>
<tr>
<td>( g_h )</td>
<td>Maximal ( I_h ) conductance</td>
<td>0.07</td>
<td>( \mu S )</td>
</tr>
<tr>
<td>( g_K )</td>
<td>Maximal ( I_K ) conductance</td>
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<td>( \mu S )</td>
</tr>
<tr>
<td>( g_{K(Ca)} )</td>
<td>Maximal ( I_{K(Ca)} ) conductance</td>
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<td>( \mu S )</td>
</tr>
<tr>
<td>( g_{leak} )</td>
<td>Maximal ( I_{leak} ) conductance</td>
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<td>( \mu S )</td>
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<td>1/sec</td>
</tr>
<tr>
<td>( k_{leak} )</td>
<td>Rate constant</td>
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<td>1/sec</td>
</tr>
<tr>
<td>( k_{leak} )</td>
<td>Rate constant</td>
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<td>1/sec</td>
</tr>
<tr>
<td>( k_{leak} )</td>
<td>Rate constant</td>
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<td>1/sec</td>
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<tr>
<td>( k_{leak} )</td>
<td>Rate constant</td>
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<td>1/sec</td>
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<tr>
<td>( E_{leak} )</td>
<td>Reversal potential of ( I_{leak} )</td>
<td>50</td>
<td>mV</td>
</tr>
<tr>
<td>( E_{Ca} )</td>
<td>Reversal potential of ( I_{Ca} )</td>
<td>140</td>
<td>mV</td>
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<td>( E_{K} )</td>
<td>Reversal potential of ( I_k )</td>
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<td>mV</td>
</tr>
<tr>
<td>( E_{leak} )</td>
<td>Reversal potential of ( I_{leak} )</td>
<td>50</td>
<td>mV</td>
</tr>
<tr>
<td>( k_h )</td>
<td>Reversal potential of ( I_h )</td>
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<td>mV</td>
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<td>( k_j )</td>
<td>( V_f ) coupling coefficient</td>
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<td>ME ( \Omega )</td>
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<tr>
<td>( k_j )</td>
<td>( V_f ) coupling coefficient</td>
<td>0.050</td>
<td>ME ( \Omega )</td>
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Results

PAIH overexpression produces large $I_h$ in PD neurons

The crustacean gene for $I_h$, channels, PAIH, was cloned from P. argus and characterized by Gisselmann et al. (2003). In the pyloric network of the lobster STG, there are two PD neurons with identical physiological properties. Therefore, we injected PAIH RNA with 4% Fast Green as an injection indicator into one PD neuron and injected Fast Green alone into the other PD neuron as an internal control. After these injections, the STG, with attached commissural and esophageal ganglia, was cultured for 5 to 7 days to allow PAIH expression before recordings were made. Our previous work has shown that the STG with the attached anterior ganglia can maintain normal oscillatory pyloric motor pattern for at least 1 week in organ culture (MacLean et al., 2003).

In control, Fast Green-injected PD neurons, the hyperpolarization-activated inward current recorded from the soma was normally very small. At $-100\text{ mV}$, the peak current was less than $-10\text{ nA}$, and the time constant for activation was $\sim 4.5\text{ sec}$ (Fig. 1A, Table 3). Current activation was voltage dependent and could be fitted by a single exponential. We extended the exponential fitting curve of the raw data to estimate the steady state of the peak current at each voltage. The activation $g/V$ curve was fitted by a Boltzmann equation (Eq. 1). The average maximal conductance was $0.15\text{ \mu S}$; the voltage for half-maximal activation ($V_0$) was $-86\text{ mV}$, whereas the slope was $10.5\text{ mV}$ (Fig. 1B, Table 3) ($n = 12$).

Compared with the control, PD cells expressing PAIH had much larger $I_h$ with significantly different biophysical properties (Fig. 1, Table 3). The average maximal conductance was increased nearly fivefold, showing successful expression of the RNA. The current was activated at significantly more depolarized potentials than the control: $V_0$ was shifted by $>10\text{ mV}$ in the positive direction ($-75\text{ mV}$), whereas the slope factor decreased by $3\text{ mV}$. In addition, the current was activated more rapidly at each voltage (Fig. 1A, Table 3); at $-100\text{ mV}$, for example, $\tau_m$ was reduced by approximately one third (Table 3). However, there were similarities in other parameters between the endogenous and the new currents: both currents had similar reversal potentials, close to $-20\text{ mV}$ (Fig. 1C), and $I_h$ in both control PD and PAIH-expressing cells were blocked by $>85\%$ by $5\text{ mm} \text{ Cs}^+$ or $300\text{ \mu M ZD7288}$ (D,E). These data indicate that PAIH encodes a standard $H$-current. When compared with the PAIH currents expressed in HEK293 cells (Gisselmann et al., 2003), $I_h$ in PD neurons was activated at a significantly more positive voltage ($V_0$ of $-75\text{ vs} -119\text{ mV}$). Thus, as seen with other $H$-current genes, the biophysical properties of the PAIH-evoked current depend markedly on the cells in which it is expressed.

PAIH overexpression does not affect $I_A$ or other outward currents

We previously showed that overexpression of the transient potassium current gene, shal, can induce a compensatory increase of $I_h$ in PD neurons, nullifying the physiological effects of shal injection (MacLean et al., 2003). To determine whether overexpression of PAIH would evoke a parallel homeostatic upregulation of $I_h$, we measured the effect of PAIH overexpression on $I_A$, as well as additional outward currents including the calcium-activated potassium current ($I_{K(Ca)}$) and the delayed rectified outward current ($I_{K(V)}$). The average peak current of $I_A$ at $+20\text{ mV}$ in control PD neurons was $379 \pm 69\text{ nA}$ ($n = 6$), whereas that in PAIH-expressing PDs was $344 \pm 64\text{ nA}$ ($n = 11$) (Fig. 2A). There were no significant differences in $I_A$ amplitude or inactivation kinetics between the control and PAIH-injected PD neurons ($p > 0.5$). In several neurons, we also checked the complete voltage dependence of activation and inactivation of $I_A$; again, there was no significant difference in these parameters between control and PAIH-expressing PD neurons (data not shown). By holding the membrane at $-40\text{ mV}, I_h$ is almost completely inactivated, and the remaining currents activated by depolarizing pulses represent the sum of $I_{K(V)}$ and $I_{K(Ca)}$. These combined outward currents also showed no significant difference between the control and PAIH-expressing PD neurons (Fig. 2B). The average peak current at $+30\text{ mV}$ was $566 \pm 64\text{ nA}$ ($n = 9$) in control and $570 \pm 66\text{ nA}$ in PAIH-expressing cells ($n = 9$). Thus, unlike our previous experiments, in which the effect of an artificial increase in $I_h$ was compensated by an endogenous upregulation of $I_h$ (MacLean et al., 2003), our experiments show that overexpression of PAIH produces a large $I_h$, but does not induce an endogenous compensatory change in outward currents.

Figure 1. Injection of PAIH cRNA produces a large $I_h$ in PD neurons. A, $I_h$ was recorded in control and PAIH-injected PDs. The cells were held at $-40\text{ mV}$, and a series of 8 sec pulses was applied from $-50$ to $-120\text{ mV}$ in 10 mV increments. Recordings were performed as described in Materials and Methods. B, Plots of $g_{I_h}$/n, versus voltage for activation of $I_h$ in control (■) and PAIH-expressing (●) PD neurons ($n = 12$ control and 19 PAIH-injected neurons). Error bars indicate SDs. C, Left, Measurement of the reversal potential from tail currents of $I_h$ in control and PAIH-expressing PD neuron. Currents were measured after a preactivating pulse to $-100\text{ mV}$ for 8 sec with a series of 4 sec pulses from $-60$ to $-20\text{ mV}$ in 5 mV increments. The tail currents were plotted against the tail voltage to determine the reverse potential. Right, Determination of $V_{rev}$ for a control cell (■) and a PAIH-expressing cell (●). D, The PAIH currents could be blocked by 5 mm $\text{Cs}^+$ and 300 $\mu\text{M ZD7288}$. 


PAIH overexpression alters the firing properties of the PD neurons

Because upregulation of $I_h$ is not compensated by outward currents in PD neurons, we expected the increased $I_h$ to change the firing properties of the injected PD neuron, relative to the control PD neuron in the same ganglion, and this turned out to be correct (Fig. 3). Compared with the control PD cell, the PAIH-expressing cell had a more depolarized minimal membrane potential, $V_{min}$, at the trough of the membrane oscillation ($-51 \pm 1.7$ mV; $n = 6$ for all of the measurements), and the oscillation amplitude was somewhat smaller than the control value ($7.4 \pm 3.2$ mV vs $10.0 \pm 3.7$ mV). The PAIH-injected PD neuron rebounded from inhibition more quickly than the control PD neuron; it had a shorter time from the $V_{min}$ to the first spike ($208 \pm 50$ vs $294 \pm 58$ msec), more spikes per burst ($7 \pm 1$ vs $5 \pm 2$), and consequently a larger duty cycle (fraction of the cycle during which the cell was spiking, $0.32 \pm 0.054$ vs $0.22 \pm 0.064$) (Fig. 3B). All of these differences were significantly different between the injected and control PD neurons ($p < 0.05$; $n = 6$ pairs).

Cesium ions block $I_h$ (Fig. 1D). When we applied 5 mM Cs$^+$ to a ganglion with one PAIH-expressing PD cell, the oscillatory frequency of the entire network significantly slowed down (from $1.15 \pm 0.38$ Hz down to $0.98 \pm 0.31$ Hz; $p < 0.01$; $n = 5$), and the oscillatory properties of PAIH-expressing cells moved back toward those seen in control, noninjected PDs (Fig. 4). The minimal membrane potential in the PAIH-expressing PD cell was hyperpolarized by $3.7 \pm 1.2$ mV (Fig. 4A), and the oscillation amplitude was increased by $2.9 \pm 1.3$ mV. As expected, the postinhibitory rebound of the cell was delayed; the time from the minimal voltage of the oscillation to the first spike was doubled ($258 \pm 98$ vs $556 \pm 172$ msec), and the duty cycle was decreased by 17% ($0.32 \pm 0.01$ vs $0.27 \pm 0.03$). These values were all significantly different from the values of the PAIH-expressing neuron before Cs$^+$ application (Fig. 4B) ($p < 0.05$; $n = 5$) and more similar to those seen in the control cells.

Dynamic-clamp simulation of $I_h$ alters the firing properties of PD neurons in the same way as PAIH overexpression

Dynamic-clamp experiments use a computer to generate and apply a modeled ionic current to a neuron in situ (Sharp et al., 1993a,b). The computer monitors the membrane potential of the neuron, calculates the conductance of the modeled current that would arise as a consequence of the changing membrane potential, and injects the appropriate current into the soma of the cell. The parameters of the modeled conductance can be altered to explore what effect the modeled conductance has on the intrinsic properties of the biological neuron under investigation. We established a dynamic-clamp system that could inject a modeled $I_h$ into PD neurons. This modeled $I_h$ had the same properties as the PAIH-expressed current in PD cells. The basic parameters we used were derived from the experiments described above: $g_{max} = 0.75 \mu$S; $V_{th} = -72$ mV; $V_{slope} = 7.5$ mV; $E_{rev} = -30$ mV; and $\tau_m = 8$ sec. The maximal currents injected were $\sim 1–2$ nA, and the output current reached steady state after 7 sec (Fig. 5A).

This artificial $I_h$ produced a similar effect on noninjected PD cells in the acutely isolated STG as overexpression of PAIH in the

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**Table 3. H-currents in control and PAIH-expressing PD cells**

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<tr>
<th></th>
<th>$g_m$ (µS)</th>
<th>$V_{1/2}$ (mV)</th>
<th>Slope (mV)</th>
<th>$\tau_m$ (at $-100$ mV)</th>
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</thead>
<tbody>
<tr>
<td>Control ($n = 12$)</td>
<td>$0.15 \pm 0.06$</td>
<td>$-86 \pm 8.2$</td>
<td>$10.5 \pm 2.1$</td>
<td>$4.48 \pm 0.89$</td>
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<tr>
<td>PAIH ($n = 19$)</td>
<td>$0.72 \pm 0.20^{**}$</td>
<td>$-75.2 \pm 6.0^{*}$</td>
<td>$7.5 \pm 1.4^{*}$</td>
<td>$2.87 \pm 0.96^{*}$</td>
</tr>
</tbody>
</table>

*Significantly different from control cells, $p < 0.05$.

**Table 3. H-currents in control and PAIH-expressing PD cells**

**Figure 2.** Overexpression of PAIH does not affect outward potassium currents. A, $I_h$ measured at $+20$ mV was not significantly different in control and PAIH-expressing PD neurons. The cells were held at $-50$ mV, and after a 400 msec deactivating prepulse to $-120$ mV, a 400 msec step to $+20$ mV was given. B, The control and PAIH-expressing cells had similar non-inactivating outward potassium currents. The cell was held at $-40$ mV, where $I_h$ is not inactivated. A series of 400 msec voltage steps were delivered from $-40$ to $+30$ mV in 10 mV increments. Drugs added to isolate the currents are described in Materials and Methods.

**Figure 3.** Overexpression of PAIH changed PD activity. A, Rhythmic activity recorded in paired control and PAIH-expressing PD neurons from the same ganglion. B, Comparison of the different firing properties in paired control (gray) and PAIH-expressing (black) PD neurons. ($n = 6$ pairs; $p < 0.05$, significantly different from control PDs). Error bars indicate SDs. The measurements of the parameters are described in Materials and Methods. Amplitude.
cultured preparations (Fig. 5B, C). Compared with the oscillation before the dynamic clamp was turned on, the cycle frequency was slightly, but significantly, increased from 1.11 to 1.16 Hz; the minimal membrane potential was depolarized by 3.5 ± 0.7 mV, and the oscillation amplitude was decreased from 22.1 ± 2.3 to 19.1 ± 2.4 mV; the time from the trough of the oscillation to the first spike was decreased from 358 ± 135 to 309 ± 105 msec, and the duty cycle was increased from 0.22 ± 0.06 to 0.26 ± 0.05 (p < 0.05; n = 5). All of these alterations in parameters were in the same direction as we found in the PAIH-expressing PDs, but were somewhat less pronounced.

We also varied the parameters of the simulated current to study their effects on the cells (n = 5). In most of our experiments, we set the activation time constant of I\textsubscript{h} at 8 sec, which was close to \(\tau_m\) for PAIH at −60 mV. When we increased \(\tau_m\) to 2 sec with the other parameters unchanged, it produced the same effects on oscillatory properties as above but sped up the time course to reach the steady state (data not shown). When we varied the maximum conductance, as we expected, all of the measured oscillatory parameters changed in parallel. For example, when the \(g_{\text{max}}\) was reduced to 0.3 \(\mu S\), the minimal membrane potential was depolarized by only 2.03 ± 0.67 mV, and the oscillation amplitude was decreased by 1.6 ± 0.5 mV. The time to the first spike was decreased to 32 ± 31 msec, the duty cycle was increased by 0.014 ± 0.012, and the firing frequency showed no change. In contrast, when \(g_{\text{max}}\) increased to 12 \(\mu S\), the minimal membrane potential was now depolarized by 4.9 ± 1.3 mV, and the oscillation amplitude was decreased by 3.9 ± 0.7 mV. The time to the first spike was decreased by 82 ± 76 msec, the duty cycle was increased by 0.036 ± 0.017, and the firing frequency increased by 0.05 ± 0.03 Hz. Shifting the value of \(V_{1/2}\) also had strong effects on the firing properties of the PD neuron. When we changed the \(V_{1/2}\) to −90 mV (near the value for endogenous \(I_h\) in the control cells), there was no effect at all of the injected \(I_h\) on the oscillatory properties of the cell, with the maximal conductance at 0.75 \(\mu S\). In contrast, when the \(V_{1/2}\) was depolarized beyond its normal value, the firing and oscillatory parameters increased accordingly (data not shown).

Theoretically modeled simple network confirms our experimental results

To further test the effects of \(I_h\) on the firing properties of PD cells, we examined the response of the two-cell model described in Materials and Methods to an \(I_h\) added to the PD cell. In this model, we had a driver cell, which mimicked the function of the pacemaker cell AB with one PD cell, and a follower cell, which represented the PAIH-evoked PD cell. In the control state of the model, the endogenous \(I_h\) is included in the leak conductance, because its activation time constant is very long relative to an oscillation. With added \(g_{\text{h}}\) set to 0, the modeled PD cell showed a firing pattern characteristic of normal PD cells in the pyloric network (Fig. 6A). Analogous to our dynamic-clamp experiments, we then added an H-current to the modeled PD neuron with constant conductance \(g_{\text{h}} = 0.07\ \mu S\), representing the \(I_h\) observed in this voltage range in the PAIH-injected cells or the \(I_h\)
properties than were seen in HEK293 cells. In particular, the V1/2 for activation was >40 mV depolarized (−119 mV in HEK293 and −75 mV in PD). This variable expression of Ih genes in different cell types has been noted previously: Qu et al. (2001, 2002) found that HCN2- and HCN4-evoked currents have very different properties when expressed in neonate versus adult cardiac ventricular cells, or in HEK293 cells. In addition, Chen et al. (2001) showed that the V1/2 for HCN1 shifted from −69 mV in an intact oocyte to −116 mV in an inside-out patch from the oocyte, and this was not merely caused by the loss of cAMP. All of these indicate that the characteristics of H-channels are profoundly modified by the cellular environment. This could arise from differences in the concentration of cAMP, the auxiliary subunits expressed by the cells, the state of phosphorylation of the channels, or cell-specific posttranslational modifications (Chen et al., 2001; Yu et al., 2001; Vargas and Lucero, 2002).

The PAIH-evoked current was also somewhat different from the endogenous Ih in PD neurons: the PAIH-encoding current had more positive V1/2 and more rapid activation kinetics (Kiehn and Harris-Warrick, 1992; Peck et al., 2000). There are several possible explanations for this discrepancy. First, although they are very closely related species, the gene(s) encoding Ih in P. interruptus may have somewhat different properties from its homolog in P. argus. Second, there may be more than one gene encoding Ih in lobsters, so PAIH alone could not produce the normal current. Third, PAIH may undergo alternative splicing, yielding splice variants with different properties. Preliminary data have shown that, in Drosophila, there are several splice variants in the Ih gene that generate H-currents with somewhat different biophysical properties; splice variants at similar positions of the gene have been also detected in Panulirus lobsters (G. Gisselmann, T. Marx, and H. Hatt, unpublished data). Finally, overexpression of PAIH may exhaust available stores of critical modulation factors or auxiliary subunits, which may normally shape the properties of the endogenous Ih. To address these possibilities, we are performing additional gene cloning from P. interruptus.

**Overexpression of PAIH does not evoke detectable homeostatic compensation by outward currents**

In a previous study, we overexpressed the transient potassium channel gene, shal, in PD neurons of the P. interruptus STG, and found that this did not significantly alter the firing properties of the injected neurons (MacLean et al., 2003). This unusual result was attributable to an activity-independent homeostatic response: the artificially evoked increase in Ih was compensated by an endogenous cellular response to upregulate Ih, which balanced and negated the physiological effects of the enhanced Ih. In this study, we wanted to determine whether overexpression of PAIH also leads to a cellular upregulation of Ih. Our current results suggest that PAIH overexpression does not significantly affect IA or the other outward currents that we measured.

There are several possible explanations for this unexpected result. First, as we mentioned above, there may be some difference between the Ih gene in P. argus and that in P. interruptus, or we may be using the incorrect splice variant of Ih that could evoke the homeostatic upregulation of Ih. Second, there may be more than one H-current gene in the lobster, and PAIH may not be the one that coregulates with Ih. In fact, the Ih evoked by shal overexpression had very different biophysical properties from both the endogenous Ih and the PAIH-evoked currents (MacLean et al., 2003; J. N. McLean, Y. Zhang, and R. M. Harris-Warrick, unpublished observations). Additional cloning efforts will help to address this possibility. Finally, it is possible that the homeostatic compensation we observed with shal RNA injections is unidirectional: that is, Ih proteins might cause an upregulation of Ih, but not vice versa. When Qu et al. (2001, 2002) overexpressed HCN2 or -4 in cardiac ventricular cells, they did not detect any
compensatory change in potassium currents. From the point of view of cellular economy, these cells may not need to regulate the consequences of enhanced \(I_h\) expression as closely as \(I_A\). We showed that subtle changes in \(I_A\) markedly alter the firing properties of pyloric neurons (Kiehn and Harris-Warrick, 1992; Harris-Warrick et al., 1995a,b; Kloppenburg et al., 1999). In our theoretical model, when we increase the maximal conductance of \(I_A\) to the value seen in \(shal\)-injected neurons, the cell is unable to oscillate normally. However, the model was much less sensitive to increases in \(I_h\): although the oscillatory properties of the cell changed as described above, it still maintained a relatively stable oscillation. Thus, it may be relatively more important to maintain homeostatic control for overexpression of \(I_A\) than of \(I_h\). This might be a reason that \(I_h\) can be a good target for neuromodulation and a significant factor shaping the firing properties of neurons.

**Overexpression of PAIH modulates the firing properties of PD neurons**

The function of \(I_h\) under normal physiological conditions is still unclear. Preliminary data suggest that \(I_h\) is small and does not play a major role in the pyloric rhythm under control conditions. Many endogenous neuromodulators of the pyloric motor pattern, such as dopamine, can shift the \(V_{th}\) of \(I_h\) in the depolarizing direction, so that \(I_h\) can become active in the physiologically relevant voltage range (Harris-Warrick et al., 1995b; Dickinson et al., 2001). Neuromodulators exert a combination of changes in synaptic function and a variety of changes in the intrinsic properties of the cells. It is always difficult to single out the function of each individual conductance. In this study, we introduced an \(I_h\) into PD neurons with a \(V_{th} \sim 10\, \text{mV}\) more depolarized than the control \(I_h\); this is similar to the \(I_h\) in the presence of dopamine, which depolarizes its \(V_{th}\) in LP neurons by 20 mV (Harris-Warrick et al., 1995b). This enhanced \(I_h\) significantly altered the firing properties of the PD neurons and the pyloric network.

In the pyloric network, there are two PD cells with identical biophysical and oscillatory properties. Normally, these cells fire synchronous bursts of action potentials. However, overexpression of PAIH on one PD cell separated it from its partner cell. The PAIH-expressing cell was more depolarized and had a smaller oscillation amplitude. Significantly, it showed a more rapid onset of spiking, with a larger duty cycle and more spikes per burst, such that the injected cell became the leading cell of the two PD neurons. We tested whether the major cause of these changes was in fact an increase in \(I_h\) by reversing them with the \(I_h\) blocker, Cs \(^+\). Five millimolar Cs \(^+\) eliminated \(I_h\) in the PAIH-expressing PD neuron and restored most of its oscillatory properties toward the control values in an uninjected PD neuron.

To further test whether the PAIH-expressed current is sufficient to cause these changes in cellular activity, we conducted dynamic-clamp experiments, modeling the additional PAIH current and using a computer to inject the calculated current into a PD neuron. In addition, we used a mathematical model of a two-cell network, comparing the response of the modeled PD with or without an additional PAIH-like current. In both experiments, application of the extra \(I_h\) evoked changes in firing properties of the PD neuron that were all in the same direction as seen after PAIH RNA injection: the modeled current depolarized the cell, reduced the oscillation amplitude and the onset time of the spikes, and increased the duty cycle. The changes in both dynamic clamp and modeled network were quantitatively less dramatic than those seen in PAIH-overexpressing cells. However, neither of our theoretical models completely represents the situation in real cells. In the dynamic-clamp experiments, the modeled current is injected only into the soma rather than distributed throughout the neuropil and in the vicinity of the spike initiation zone, in which we expect the PAIH channels to be expressed. Our previous studies have shown that the effects of ionic currents on oscillatory properties are very sensitive to the location of the currents relative to the spike initiation zone (MacLean, Zhang, R. A. Ricardo, R. Casey, J. Guckenheimer, and Harris-Warrick, unpublished data). The mathematical model of the two-cell network was not intended as an accurate representation of the pyloric circuit. Instead, we used it as a computational test bed for examining the effects of adding an \(I_h\) to a PD cell driven by rhythmic oscillations. In this model, the PD cell was represented as a single compartment that lacks the structural complexity of the real neurons. Because of these limitations, we can only show that the responses of the PD cell in the dynamic-clamp and theoretical simulations are qualitatively consistent over a number of different parameters with our data from PAIH-injected PD cells. Overall, these simulations support the hypothesis that alteration of \(I_h\) by itself is sufficient to explain the effects of PAIH RNA injection; there is no need to consider additional compensatory current changes. This is consistent with the lack of compensation by \(I_A\), \(I_{K_{Ca}}\), and \(I_{K_{Na}}\).

In conclusion, our study has demonstrated that overexpression of an \(I_h\) gene, PAIH, causes significant changes in the membrane potential and firing properties of the PD neurons within the pyloric network. Overexpression of PAIH does not alter the expression of \(I_A\) or other outward currents, so the homeostatic compensation we previously observed after upregulation of \(I_h\) is not bidirectional.

**References**


Santoro B, Grant SG, Bartsch D, Kandel ER (1997) Interactive cloning with the SH3 domain of N-src identifies a new brain specific ion channel protein, with homology to Eag and cyclic nucleotide-gated channels. Proc Natl Acad Sci USA 94:14815–14820.


